Signal-induced site-specific phosphorylation targets IκBα to the ubiquitin–proteasome pathway

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The transcription factor NF-κB is sequestered in the cytoplasm by the inhibitor protein IκBα. Extracellular inducers of NF-κB activate signal transduction pathways that result in the phosphorylation and subsequent degradation of IκBα. At present, the link between phosphorylation of IκBα and its degradation is not understood. In this report we provide evidence that phosphorylation of serine residues 32 and 36 of IκBα targets the protein to the ubiquitin–proteasome pathway. IκBα is ubiquitinated in vivo and in vitro following phosphorylation, and mutations that abolish phosphorylation and degradation of IκBα in vivo prevent ubiquitination in vitro. Ubiquitinated IκBα remains associated with NF-κB, and the bound IκBα is degraded by the 26S proteasome. Thus, ubiquitination provides a mechanistic link between phosphorylation and degradation of IκBα.

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NF-κB and other members of the Rel family of transcriptional activator proteins regulate the expression of the type I human immunodeficiency virus (HIV) as well as a large number of cellular genes that play essential roles in immune and inflammatory responses (for review, see Grilli et al. 1993; Baeuerle and Henkel 1994; Siebenlist et al. 1994; Thanos and Maniatis 1995). The transcriptional activities of Rel proteins are highly regulated in most cell types by a mechanism that involves specific association between heterodimeric Rel complexes and a family of monomeric inhibitor proteins designated IκB (Baeuerle and Baltimore 1988; for review, see Beg and Baldwin 1993; Gilmore and Morin 1993). Members of this inhibitor family share a structural domain comprised of five to six ankyrin-like repeats. The best-characterized IκB protein, IκBα, binds to the p50 (NF-κB1)/p65 (RelA) heterodimer of NF-κB and masks the nuclear localization signals of these proteins (Beg et al. 1992; Ganchi et al. 1992; Henkel et al. 1992; Zabel et al. 1993). When cells are exposed to a variety of NF-κB inducers such as lipopolysaccharide (LPS), phorbol esters, tumor necrosis factor-α (TNFα), and interleukin-1 (IL-1), IκBα is rapidly phosphorylated and degraded, and NF-κB translocates to the nucleus where it activates gene expression (Beg et al. 1993; Brown et al. 1993; Cordle et al. 1993; Henkel et al. 1993; Rice and Ernst 1993; Sun et al. 1993, 1994a).

An alternative pathway for regulating the activity of NF-κB involves proteolytic processing of the p105 precursor of p50 subunit of NF-κB (Blank et al. 1991; Fan and Maniatis 1991; Mellits et al. 1993; Mercurio et al. 1993; Donald et al. 1995). The p105 precursor contains p50 at its amino terminus and an IκB-like sequence with ankyrin repeats at its carboxyl terminus. Unprocessed p105 can associate with p65 and other members of the Rel family to form inactive heterodimeric complexes that are sequestered in the cytoplasm (Capobianco et al. 1992; Liou et al. 1992; Neumann et al. 1992; Rice et al. 1992; Mercurio et al. 1993). Processing of p105 results in degradation of the IκB-like carboxyl terminus and the production of the transcriptionally active p50/Rel protein heterodimer.

Recently, p105 processing was shown to require the ubiquitin–proteasome pathway (Palombella et al. 1994). This pathway requires ATP and the covalent conjugation of target proteins with multiple ubiquitin molecules (for review, see Goldberg 1992; Hershko and Ciechanover 1992; Jentsch 1992). Ubiquitination occurs in a three-step process. In the first step, ubiquitin is activated by a ubiquitin-activating enzyme (E1), and in the second step, the activated ubiquitin is transferred to a ubiquitin carrier protein (E2). In the final step, ubiquitin–protein ligase (E3) catalyzes the covalent attachment of ubiquitin

to the target protein. Additional ubiquitins are then thought to be added by a processive mechanism to form the multiubiquitin chain (for recent review, see Ciechanover 1994). The multiubiquitinated proteins are then rapidly degraded by the 26S proteasome.

The 26S proteasome consists of a 20S multicatalytic protease complex and additional regulatory subunits that are required for the recognition and degradation of multiubiquitinated proteins (for review, see Rechsteiner et al. 1993; Peters 1994). The NF-κB1 p105 protein is ubiquitinated in vitro, and ubiquitination is required for in vitro processing by purified 26S proteasome (Palombella et al. 1994). In addition, p105 processing in vitro and in vivo is blocked by peptide aldehyde inhibitors of the proteasome. The degradation of IκBα is also blocked by such inhibitors, but this process has not yet been shown to require ubiquitination (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995).

Although the signal transduction pathways leading to the activation of NF-κB are not well understood, these pathways culminate in the phosphorylation of IκB, p105, and p65 (Beg et al. 1993; Brown et al. 1993; Mellits et al. 1993; Naumann and Scheidereit 1994; Sun et al. 1994b; Donald et al. 1995). Initially, phosphorylation of IκBα was thought to promote its dissociation from NF-κB and its subsequent degradation (for discussion, see Beg and Baldwin 1993; Beg et al. 1993). This conclusion was consistent with the observation that tosyl-Phe-chloromethylketone (TPCK) and other alkylating agents block the degradation of IκBα and the activation of NF-κB (Henkel et al. 1993; Mellits et al. 1993). However, more recent studies have shown that these inhibitors actually prevent the signal-dependent phosphorylation of IκBα and have no direct effect on proteasome function (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). In contrast, the presence of proteasome inhibitors leads to the accumulation of phosphorylated IκBα bound to NF-κB (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). These findings suggest that phosphorylation leads to the degradation of IκBα by the proteasome, without inducing its dissociation from NF-κB.

Recently, serine residues 32 and 36 in IκBα have been shown to be required for IκBα phosphorylation and degradation in response to TNF-α, phorbol 12-myristate 13-acetate (PMA), and ionomycin (Brockman et al. 1995; Brown et al. 1995) or the Tax protein of the type I human T-cell leukemia virus (HTLV-1; Brockman et al. 1995). However, the mechanisms by which phosphorylation leads to the degradation of IκBα are not understood. In this paper we show that IκBα is ubiquitinated in vivo and in vitro and that ubiquitination is required for degradation by the 26S proteasome. In addition, we demonstrate that mutations in IκBα that prevent its phosphorylation and degradation in vivo block ubiquitination in vitro. Together, these findings indicate that the signal-dependent phosphorylation of IκBα targets the cytoplasmic inhibitor to the ubiquitin–proteasome pathway.

Results

Inducible phosphorylation and ubiquitination of IκBα in vivo

To determine whether IκBα is ubiquitinated upon phosphorylation in vivo, we sought conditions that result in the stabilization of the hyperphosphorylated form of IκBα that is rapidly degraded during cellular activation (for review, see Siebenlist et al. 1994). Previous studies demonstrated that the proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) blocks TNFα-induced degradation of IκBα and leads to the accumulation of phosphorylated IκBα (Palombella et al. 1994). However, only a small fraction of endogenous IκBα remains phosphorylated under these conditions, attributable presumably to the action of endogenous phosphatases. Calyculin A and okadaic acid are phosphatase inhibitors that induce NF-κB by maintaining phosphorylation and degradation of IκBα (Thevenin et al. 1990; Menon et al. 1993; Lin et al. 1995). We therefore attempted to accumulate phosphorylated IκBα in the Jurkat T-cell line using the combination of MG132 and calyculin A. Jurkat cells were treated with 40 μM MG132 alone (Fig. 1A, lane 2), with 0.3 μM calyculin A alone (lane 3), or with both inhibitors (lane 4), and the phosphorylation of IκBα analyzed in a Western blot using a polyclonal antibody against the carboxyl terminus of IκBα. Treatment with MG132 alone did not affect the level of unphosphorylated IκBα under these conditions (although IκBα is known to be basally phosphorylated (for example, Brown et al. 1995), we will refer to unstimulated IκBα as unphosphorylated). Treatment with calyculin A alone resulted in the phosphorylation and degradation of IκBα. In contrast, phosphorylated IκBα accumulated when cells were treated with both inhibitors. The calpain inhibitor MG102 (40 μM), which completely inhibits calpain activity but does not inhibit the proteasome at this concentration, did not lead to accumulation of phosphorylated IκBα in the presence of calyculin A (data not shown). These results indicate that calyculin A induces the phosphorylation-dependent degradation of IκBα and that the proteasome is required for this process.

To determine whether ubiquitination of IκBα occurs in vivo, we prepared cell extracts at different times after treatment of Jurkat cells with calyculin A in the presence of MG132 and then analyzed the samples by Western blotting using antibodies against IκBα. The extracts were prepared in the presence of SDS (0.1%) and *N*-ethylmaleimide (NEM, 5 mM) to inhibit isopeptidase activities that may otherwise affect the detection of ubiquitinated proteins. As shown in Figure 1B, a ladder of high molecular mass proteins accumulated following stimulation with calyculin A/MG132 (lanes 4–9). The molecular mass increments of these ladders were ~8.5 kD, which is the size of ubiquitin. Ubiquitination of IκBα peaked at 5–15 min following stimulation (lanes 4–6)

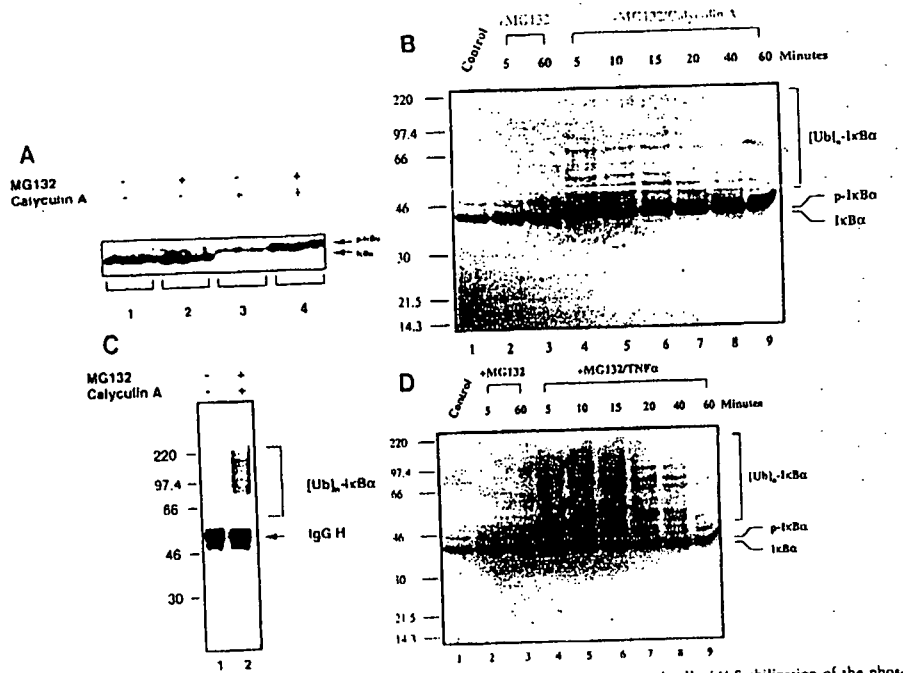

Figure 1. Induced phosphorylation and ubiquitination of IκBα in calyculin A or TNFα-treated cells. (*A*) Stabilization of the phosphorylated form of IκBα by MG132. Jurkat T cells were pretreated with 40 μM of MG132 (lanes *2*,*4*) or DMSO (a diluent for MG132, lanes *1*,*3*) for 30 min before incubation with 0.3 μM of calyculin A (lanes *3*,*4*) or DMSO (lanes *1*,*2*) for an additional 30 min. Cytoplasmic extracts were prepared, fractionated by SDS-PAGE, and analyzed by Western blot using a rabbit polyclonal antibody against the carboxyl terminus of IκBα (amino acids 297–317, c-21). The phosphorylated form of IκBα is designated p-IκBα. (*B*) Induced ubiquitination of IκBα by calyculin A. Jurkat T cells were pretreated with MG132 (40 μM, lanes 2–9) or DMSO (lane *1*) for 30 min before addition of calyculin A (0.3 μM, lanes 4–9) or DMSO (lanes 2,3). Cell extracts were prepared at 5 min (lanes 2,4), 10 min (lane 5), 15 min (lane 6), 20 min (lane 7), 40 min (lane 8), and 60 min (lanes 3,9) after adding calyculin A or DMSO. The extraction buffers contained RIPA/0.1% SDS plus 5 mM *N*-ethylmaleimide (NEM). The extracts were then subjected to Western blot analysis as described in *A*. (*C*) Jurkat T cells were treated as described in lanes *1* and *4* in *A*, except that cytoplasmic extracts were first immunoprecipitated with an antibody against the carboxyl terminus of IκBα (see Materials and methods). The immunoprecipitates were then eluted with 0.1 M Capso (pH 11.2), and the eluates were separated by SDS-PAGE, followed by Western blotting with a rabbit polyclonal antibody (anti-Ub) that specifically recognizes ubiquitin conjugates. The arrow marked IgG H indicates rabbit immunoglobulin heavy chain in the immunoprecipitates that cross-reacts with the secondary antibody (alkaline phosphatase–conjugated anti-rabbit Fcl. (*D*) Induced ubiquitination of IκBα by TNFα. Experiments were carried out as described in *B*, except that calyculin A was replaced with TNFα (10 ng/ml).

and then decreased by 40–60 min (lanes 8,9), possibly because of residual activities of proteasome and isopeptidases. Treatment of Jurkat cells with MG132 alone did not lead to significant accumulation of ubiquitinated IκBα (lane 2). Longer treatment with MG132 (60 min plus 30 min of pretreatment, lane 3) led to the appearance of very faint bands corresponding to ubiquitinated IκBα, suggesting that ubiquitination may also be involved in basal turnover of IκBα.

To further demonstrate that calyculin A induces ubiquitination of IκBα in vivo, we immunoprecipitated IκBα from control and MG132/calyculin A-treated Jurkat cell extracts with an IκBα antibody. We then performed a Western blot analysis on the immunoprecipitated proteins using a polyclonal antibody against ubiquitin (Fig. 1C). The specificity of these antibodies has been documented extensively, and the antibodies have been widely used to detect ubiquitinated proteins (Haas and Bright 1985; Lowe and Mayer 1990). The anti-ubiquitin antibody detected high molecular mass proteins in the calyculin A/MG132-treated extracts. These molecular masses ranged from 60 to 200 kD, consistent with a typical pattern seen upon multiubiquitination of proteins. Taken together, these results suggest that calyculin A induces the phosphorylation-dependent ubiquitination of IκBα in vivo.

Because calyculin A is not a natural inducer of NF-κB activation, it is possible that induced ubiquitination of IκBα is an unusual effect of calyculin A. To address this possibility, we performed an experiment similar to that described in Figure 1B, except that calyculin A is replaced by TNFα (Fig. 1D), a natural inducer of NF-κB. Strikingly, TNFα not only induces hyperphosphorylation of IκBα in the presence of MG132 but also induces multiubiquitination of IκBα (lanes 4–9). The kinetics of induction by TNFα is similar to the induction by calyculin A. We conclude that IκBα is rapidly phosphorylated and ubiquitinated following stimulation by inducers of NF-κB.

In vitro-translated IκBα is ubiquitinated in HeLa cell extracts, and the ubiquitinated IκBα remains associated with NF-κB

Next, we carried out experiments to determine whether the phosphorylation and ubiquitination of IκBα could be induced in vitro by the phosphatase inhibitor okadaic acid. Like calyculin A, okadaic acid has been shown previously to potently activate NF-κB in vivo (Thevenin et al. 1990). Preliminary experiments revealed that incubation of HeLa cell cytoplasmic extracts in the presence of MgATP and okadaic acid led to a time-dependent phosphorylation and ubiquitination of endogenous IκBα (data not shown). We extended this study to in vitro-translated IκBα so that IκBα mutants could be examined (see below). We prepared ^{35}S-labeled IκBα by coupled in vitro transcription/translation of IκBα mRNA in wheat germ extracts. The in vitro-translated IκBα was then incubated in the HeLa cell cytoplasmic extract in the presence of MgATP, ubiquitin, okadaic acid, and ubiquitin aldehyde (Ubal), an isopeptidase inhibitor that prevents the breakdown of ubiquitin conjugates. As shown in Figure 2A, there was a time-dependent accumulation of high molecular mass species characteristic of multiubiquitinated IκBα, with a concomitant decrease in unconjugated IκBα. The molecular weights of the slowly migrating species range from 60 to >200 kD, consistent with the addition of from 10 to >20 ubiquitin molecules (8.5 kD) to each molecule of IκBα (37–41 kD, depending on phosphorylation states). The unconjugated IκBα present during the ubiquitination reaction is a doublet, and as described below, the upper band is likely phosphorylated IκBα. The high molecular mass species can be immunoprecipitated by anti-ubiquitin antisera (Fig. 2B, lane 2). In addition, when glutathione *S*-transferase–ubiquitin (GST–Ub) was used to substitute for ubiquitin, the high molecular mass species could be precipitated by glutathione–Sepharose (data not shown). Furthermore, the high molecular mass species could be converted to lower molecular mass forms upon treatment with isopeptidase T (data not shown), which cleaves lysine-48 linked isopeptide bonds between ubiquitin molecules (Chen and Pickart 1990). These observations, together with data shown below, clearly show that the high molecular mass species are multiubiquitinated IκBα.

The conditions required for ubiquitination of IκBα in vitro were examined in the experiment of Figure 2C. Both IκBα (lanes 1–6) and FLAG epitope-tagged IκBα (F-IκBα, lanes 7–12) were tested in this experiment. The latter form of IκBα was examined for comparison to a series of FLAG-tagged IκBα mutants, which were examined previously in vivo (Brockman et al. 1995; see below and Fig. 3). With both the native and FLAG-tagged IκBα proteins, phosphorylation and ubiquitination were dependent on the presence of okadaic acid (lanes 2 and 5, 8 and 11), and both reactions were abolished by the addition of EDTA (lanes 6,12). The upper bands labeled p-IκBα and p-F-IκBα are phosphorylated forms of IκBα, as these bands could be converted to the lower bands after treatment with calf intestine alkaline phosphatase (CIAP, data not shown). The presence of Ubal was necessary for multiubiquitination of IκBα (lanes 4,10). Without Ubal, only small amounts of low molecular mass conjugates were observed, probably because of the action of isopeptidases in the extract. Multiubiquitination of IκBα was also inhibited by methylated ubiquitin (MeUb, lanes 3,9), which prevents the formation of multiubiquitin chains (Hershko et al. 1991). Because of the presence of competing endogenous ubiquitin in the HeLa cell extracts, MeUb functions as a ubiquitin chain terminator that leads to the generation of low levels of low molecular mass conjugates. The fact that the formation of high molecular mass conjugates is dependent on Ubal and inhibited by MeUb provides strong evidence that the high molecular mass conjugates are multiubiquitinated IκBα. Addition of GST–c-Rel protein did not affect the in vitro ubiquitination reaction in HeLa cell extract (data not shown), presumably because the in vitro-translated IκBα associates with excess endogenous NF-κB proteins present in the extract (see below). Taken together, these results show that IκBα can be ubiquitinated in vitro. Moreover, ubiquitination requires the presence of an inducing agent, such as okadaic acid.

To determine whether the IκBα ubiquitinated in HeLa cell extracts is associated with NF-κB, the reaction mixture (Fig. 3, lane 1) was precipitated with an antibody against RelA (lane 2). Both ubiquitinated and unconjugated IκBα were present in the anti-RelA precipitates, indicating that both are associated with NF-κB. The ratio of ubiquitinated IκBα to unconjugated IκBα in the immunoprecipitates is similar to that in the reaction mixture prior to immunoprecipitation, suggesting that both forms of IκBα associate with NF-κB with similar affinity.

Serine residues 32 and 36 are necessary for ubiquitination of IκBα in vitro

Serine residues 32 and 36 of IκBα are required for the phosphorylation and degradation of IκBα in vivo (Brockman et al. 1995; Brown et al. 1995). However, a mechanistic link between phosphorylation and degradation had

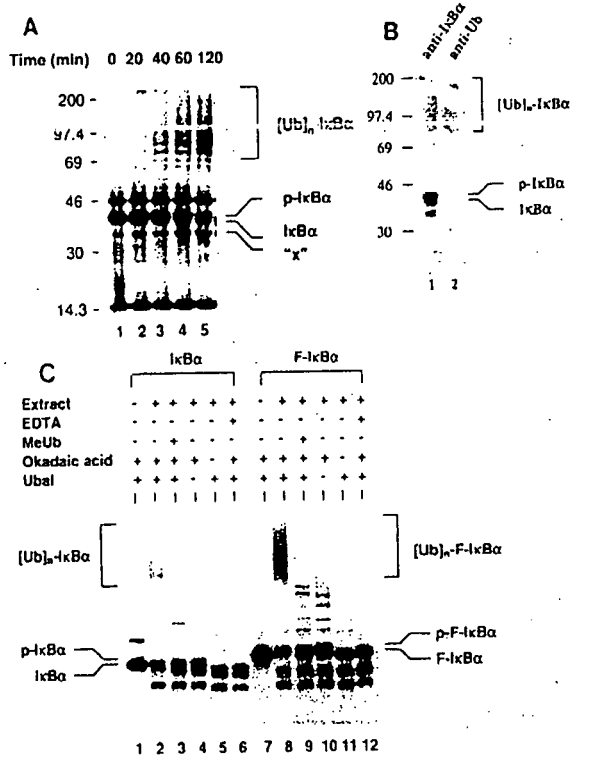

Figure 2. Induced phosphorylation and ubiquitination of IκBα in vitro. (*A*) Time course of ubiquitination. IκBα was in vitro-translated in wheat germ extract (Promega TNT system) in the presence of [^{35}S]methionine. The labeled IκBα was then incubated in HeLa extracts at 37°C in the presence of Mg-ATP (2 mM), ubiquitin (1 mg/ml), okadaic acid (3.3 μM), and Ubal (3 μM). An aliquot of the reaction was then quenched at the indicated times in SDS sample buffer, followed by SDS-PAGE and fluorography. (*B*) Double immunoprecipitation with IκBα and ubiquitin antibodies. Ubiquitinated IκBα was synthesized as described in *A*, and the reaction mixture was precipitated with IκBα antibody (lane *1*). An aliquot of the immune complex was then boiled for 5 min in the presence of 0.5% SDS, and the liberated ubiquitinated IκBα was then precipitated again with anti-ubiquitin antibody (lane *2*). The samples were analyzed by SDS-PAGE followed by fluorography. (*C*) Conditions required for phosphorylation and ubiquitination of IκBα in vitro. Both IκBα (lanes *1–6*) and FLAG-epitope-tagged IκBα (F-IκBα, lanes *7–12*) were ^{35}S- labeled by in vitro translation and used as substrates for ubiquitination. The reaction conditions were as described in *A*, except for the following: Lanes *3* and *9* received MeUb (1.3 mg/ml) instead of Ub; lanes *4* and *10* lacked Ubal in the reaction; lanes *5* and *11* lacked okadaic acid in the reaction; lanes *6* and *12* received EDTA (40 mM) instead of Mg-ATP. After incubation at 37°C for 90 min, IκBα and F-IκBα were immunoprecipitated with IκBα antisera (c-21) and then analyzed by SDS-PAGE. In *A–C*, the bands below IκBα are probably nonspecific partial proteolytic cleavage products of IκBα, as the generation of these bands is not affected by EDTA, MeUb, okadaic acid, or Ubal (see *C*).

not been established. We therefore tested a series of phosphorylation-defective mutants of IκBα in the in vitro ubiquitination assay. Wild-type and mutant IκBα proteins tagged at their amino termini by the FLAG epitope (Brockman et al. 1995) were produced by in vitro translation (Fig. 4A). These same mutants were analyzed previously for their effects on the inducible degradation of IκBα in vivo (Brockman et al. 1995).

The first 36 amino-terminal amino acids have been deleted in the ΔN mutant, whereas the S32A and S36A mutants are serine to alanine substitutions at positions 32 and 36, respectively. The S32A/S36A mutant is a double serine to alanine substitution at positions 32 and 36. All of these mutant proteins are stable when expressed in cells treated with TNFα, PMA/ionomycin, or in the presence of the HTLV Tax protein. Morever, they retain their ability to associate with RelA, and are dominant negative inhibitors of NF-κB activation (Brockman et al. 1995). The S32E and S36E mutants are serine to glutamic acid substitutions, which are designed to mimic the negative charge of phosphorylation (note mobility differences on SDS–polyacrylamide gel; Fig. 4B). These substitutions restore the ability of the mutant IκBα to be degraded in response to inducing agents in vivo (Brockman et al. 1995). The ΔC mutant lacks 75 amino acids at the carboxyl terminus of IκBα. This mutation removes a carboxy-terminal PEST sequence, as well as a putative sixth ankyrin repeat. The sixth ankyrin repeat has been shown to be required to bind NF-κB (Ernst et al. 1995). In addition, deletion of the PEST sequence has been shown to stabilize IκBα in vivo (Miyamoto et al. 1994; Brockman et. al., 1995; Brown et al. 1995). PEST sequences in other proteins have been implicated in protein degradation (Rogers et al. 1986). (Note that this ΔC mutant is different from another recently described ΔC mutant, which is missing only 41 residues at the carboxyl terminus, and can associate with RelA/p65; Brown et al. 1995).

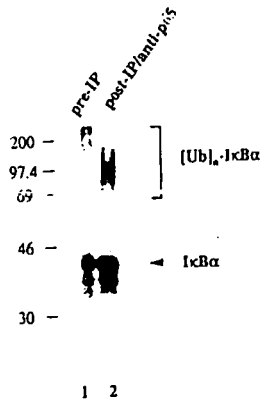

Figure 3. Association of ubiquitinated IκBα with NFκB. Conditions for the synthesis of ubiquitinated IκBα were as described in Fig. 2A except that the reaction was carried out at 37°C for 2 hr. Ten percent of the reaction mixture was saved for SDS-PAGE analysis (lane *1*), and IκBα in the remaining mixture was coimmunoprecipitated with RelA using antisera against RelA/p65 (see Materials and methods). The precipitates were boiled in SDS sample buffer, followed by SDS-PAGE and fluorography (lane *2*). An amount equivalent to ~50% of the initial reaction volume was loaded in lane *2*, which is fivefold greater than that loaded in lane *1*. The bulk of the high molecular mass conjugates in lane 2 is approximately 100 kD, as opposed to 200 kD seen in lane *1*, and this may be attributable to "trimming" by isopeptidases during the immunoprecipitation.

Analysis of these mutants in the in vitro ubiquitination assay revealed an excellent correlation between their ability to be degraded in vivo in response to inducers and their ability to be ubiquitinated in vitro (Fig. 4C). Specifically, ΔN (lane 12), S32A (lane 14), S36A (lane 16), and S32A/S36A (lane 18), which escape signal-dependent degradation in vivo (Brockman et al. 1995), are also not ubiquitinated in vitro. In contrast, wild-type IκBα (lane 11), S32E (lane 15), and S36E (lane 17) are all ubiquitinated in this assay, consistent with their functional phenotype in vivo (Brockman et al. 1995). The ΔC mutant protein was ubiquitinated in vitro (lane 13), but its degradation is not enhanced in vivo following stimulation of cells. However, the basal turnover of this mutant is indistinguishable from that of the wild type (data not shown). Given that this ΔC mutant does not associate with RelA, it is likely that its behavior reflects signal-independent (basal) turnover of free IκBα (albeit at low level) in the cell. It is possible that ubiquitination is also involved in the basal turnover of free IκBα and that the carboxy-terminal sequence including the PEST region is not required for ubiquitination of free IκBα.

A shorter exposure of the film shown in Figure 4, A and C, showed a remarkable correlation between phosphorylation and ubiquitination of these mutants in HeLa extracts (Fig. 4B,D). A clear difference in electrophoretic mobility of the unconjugated IκBα mutants following incubation in the HeLa cell extracts can be observed. For example, after incubation in the okadaic acid-supplemented extracts, the unconjugated wild-type IκBα exhibits a slightly slower migrating band in addition to the band observed prior to incubation (cf. Fig. 4B, lane 1, and 4D, lane 11). This "mobility shift" phenomenon is not observed in ΔN (lanes 2,12) and S32A/S36A mutants (lanes 8,18). This slower migrating band is attributable to phosphorylation of IκBα. The S32A and S36A mutant proteins were also phosphorylated during the reaction (lanes 4 and 14, 6 and 16), probably because the adjacent unaltered serine residues (S36 and S32, respectively) can serve as phosphoryl group acceptors. The S32E and S36E mutants both migrate more slowly than S32A, S36A, or S32A/S36A even before the ubiquitin conjugation reaction (lanes 5 and 15, 7 and 17), an indication that the negative charges on S32E and S36E decrease the mobility of both proteins. The mobility of the S32E and S36E mutants did not change appreciably after the reaction, suggesting that additional phosphorylation at the alternate serine residue S36 and S32, respectively, did not change significantly the electrophoretic mobility of IκBα. These results demonstrate that phosphorylation of the IκBα mutants in HeLa cell extracts correlates with their ability to be ubiquitinated.

The alkylating agent TPCK and the antioxidant pyrolidinedithiocarbamate (PDTC) have been widely used to inhibit the induced degradation of IκBα (Beg et al. 1993; Henkel et al. 1993; Sun et al. 1993). These agents act by inhibiting the phosphorylation of IκBα. As shown in Figure 4, C and D, TPCK (50 μM) also inhibited phosphorylation and ubiquitination of IκBα in HeLa cell extracts (lane 9). In contrast, PDTC (50 μM) did not inhibit either phosphorylation or ubiquitination of IκBα in this assay (lane 10), probably because PDTC acts upstream of the okadaic acid activation step or, alternatively, generation of reactive oxygen intermediates (ROI) was not faithfully reproduced in this system. Taken together, there was an excellent correlation between phosphorylation and ubiquitination of IκBα in vitro. We conclude that both serine residues 32 and 36 are required for ubiquitination of IκBα in vitro, most likely through direct phosphorylation of these sites (see discussion).

Ubiquitinated IκBα bound to NF-κB is degraded by the 26S proteasome

To determine whether ubiquitination of IκBα is required for degradation by the 26S proteasome in vitro, we compared the fate of conjugated and unconjugated IκBα when incubated with purified 26S proteasome. IκBα labeled with [^{35}S]methionine was produced by in vitro translation and incubated in HeLa cell extracts in the presence or absence of EDTA. (EDTA blocks ubiquitination and therefore serves as a control; see Fig. 2C). The IκBα/NF-κB complex formed in vitro was then immunopre-

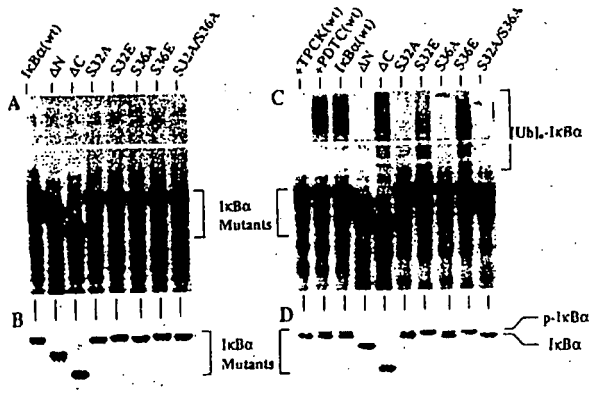

Figure 4. Mutations that prevent the phosphorylation of IκBα in vivo also abolish ubiquitination in vitro. (*A*) In vitro-translated IκBα mutants. IκBα mutants were translated in wheat germ extracts (Promega TNT system) in the presence of [^{35}S]methionine. The translation products were analyzed by SDS-PAGE and fluorography. (*B*) A shorter exposure of *A* showing the IκBα mutants before ubiquitination reactions. (*C*) Ubiquitination assays. IκBα mutants shown in *A* were incubated in HeLa cell extracts at 37°C for 1 hr under conditions described in Fig. 2A. The reaction mixtures were then subjected to SDS-PAGE and fluorography. In lanes 9 and *10*, TPCK (50 μM) and PDTC (50 μM) were added to the reactions containing wild-type IκBα, respectively. (*D*) A shorter exposure of *C*, showing the phosphorylation properties of the remaining unconjugated IκBα mutants. p-IκBα is the phosphorylated form of IκBα or IκBα bearing glutamic acid substitutions at position 32 or 36.

cipitated with an anti-RelA antibody (see above and Fig. 3). Both unconjugated and ubiquitinated IκBα were isolated as a complex with NF-κB. The immunoprecipitated IκBα proteins were then separated by SDS-PAGE as shown in the fluorograph of Figure 5A (lanes 1,2). Quantitation of the data by PhosphorImager analysis showed that ubiquitin-conjugated IκBα contained 67% of the total radioactivity, and the remaining 33% was unconjugated IκBα. When both conjugated and unconjugated IκBα were incubated with purified 26S proteasomes (23 nM) in the presence of Mg and ATP, a significant (47%) reduction in the level of conjugated IκBα was observed (cf. lanes 2 and 4), whereas the amount of unconjugated IκBα did not significantly change (cf. lanes 1 and 3). The level of unconjugated IκBα in lane 4 is slightly higher than that in lane 2, probably because of isopeptidase activities associated with the 26S proteasome (Eytan et al. 1993). To directly measure the degradation of ubiquitinated IκBα by the 26S proteasomes, the degradation products were separated from undegraded IκBα by trichloroacetic acid (TCA) precipitation, and the TCA-soluble radioactivity was determined. The percentage of conjugate degradation, taking into account the unconjugated IκBα present in the conjugate sample, is plotted in Figure 5B. Ubiquitinated IκBα is efficiently degraded by the 26S proteasome (17 nM, open square). Approximately 19% of the substrate was degraded within 5 min, and by 1 hr, 51% of the conjugates was degraded. Inclusion of EDTA in the degradation reaction abolished degradation of the conjugates (solid square), indicating that the 26S proteasome-catalyzed degradation is Mg-ATP dependent. The 20S proteasome, which functions as the proteolytic core of the 26S complex, did not degrade ubiquitinated IκBα (open triangle). This is consistent with the role of the 26S proteasome in degrading ubiquitinated substrates. Similarly, no degradation of the conjugates occurred in the absence of the 26S proteasome (solid triangle). Importantly, unconjugated IκBα was not degraded by the 26S proteasome (open diamond). These results clearly demonstrate that ubiquitination of IκBα targets the protein for degradation by the 26S proteasome. Thus, it appears that IκBα is not only phosphorylated and ubiquitinated while associated with NF-κB, but ubiquitinated IκBα also serves as a substrate for the 26S proteasome when complexed to NF-κB.

Discussion

The transcription factor NF-κB is activated in response to a large number of distinct extracellular signals, all of which result in the phosphorylation of IκB proteins (for review, see Siebenlist et al. 1994). Recently, serine residues 32 and 36 were shown to be required for phosphorylation and degradation of IκBα, and activation of NF-κB (Brockman et al. 1995; Brown et al. 1995). In this paper we have shown that IκBα is ubiquitinated in response to stimulation both in vivo and in vitro, and that phosphorylation of IκBα is required for ubiquitination. In addition, we show that ubiquitinated IκBα is degraded by the 26S proteasome in vitro. The latter observation is consistent with previous observations showing that proteasome inhibitors block the degradation of IκBα in vivo (Finco et al. 1994; Miyamoto et al. 1994; Palombella et al. 1994; Traenckner et al. 1994; Alkalay et al. 1995; DiDonato et al. 1995; Lin et al. 1995). Although these inhibitors were shown to act on purified proteasomes, they can also inhibit other proteases, such as calpains and cathepsins. A series of peptide–aldehyde inhibitors with different potencies (IC_{50}s) against the purified proteasome in vitro (Rock et al. 1994) were tested for their

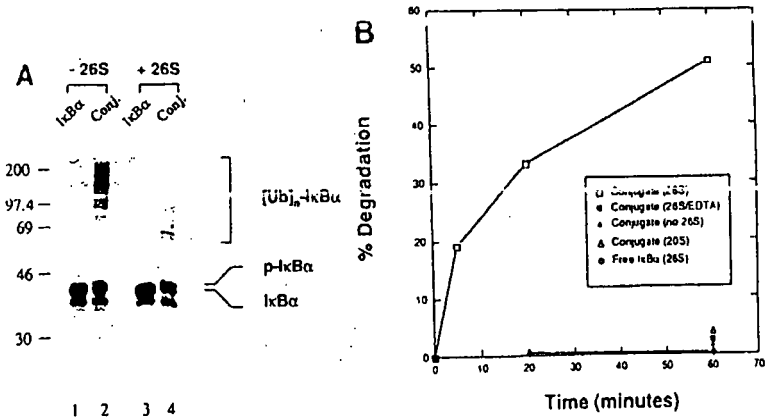

Figure 5. Degradation of ubiquitinated IκBα by the 26S proteasome. (*A*) In vitro-translated ^{35}S-labeled IκBα was incubated in HeLa extracts at 37°C for 2 hr under ubiquitination conditions (see Materials and methods), except that either Mg-ATP (lanes *2,4*) or EDTA (lanes *1,3*) was added to the reaction. The reaction mixtures were then immunoprecipitated by an antibody against RelA under conditions that allow coprecipitation of conjugated (lanes *2,4*) or unconjugated (lanes *1,3*) IκBα. The immunoprecipitates were then used directly for the degradation assay by the 26S proteasome. Lanes *1* and *2* are minus (−) 26S; lanes 3 and 4 are plus (+) 26S (23 nM). The degradation reactions were carried out at 37°C for 1 hr in the presence of Mg·ATP, and the reaction mixtures were separated by SDS-PAGE, followed by fluorography. (*B*) Ubiquitinated IκBα in the immunoprecipitates described in *A* (lane 2) was incubated with 26S proteasome (17 nM) at 37°C in the presence of Mg·ATP (□). At indicated time points, an aliquot of the reaction was precipitated by 10% TCA. The TCA-soluble radioactivity was then determined by liquid scintillation counting. Similarly, degradation of unconjugated (free) IκBα was also determined (○). In other reactions, 40 mM EDTA was added (■), 26S proteasome was omitted (▲), and 20S proteasome (49 nM) was added (△) instead of the 26S proteasome.

ability to inhibit NF-κB in vivo (Palombella et al. 1994; Read et al. 1995). The rank order potencies of these compounds in vitro and in vivo were in excellent agreement. In contrast, other calpain and cathepsin inhibitors, even at high concentrations, did not block NF-κB activation. Thus, it seems likely that the antagonistic effects of these agents on NF-κB activation derive from their inhibitory activity on the proteasome. Moreover, in related studies it was found that lactacystin, a highly specific inhibitor of the proteasome (Fenteany et al. 1995), also prevents the processing of p105, the degradation of IκBα, and the activation of NF-κB in vivo (J. Hagler, O.J. Rando, G. Fenteany, S.L. Schreiber, and T. Maniatis, unpubl.). In addition, a new class of synthetic proteasome inhibitors, which do not affect any other known cellular proteases, also blocks IκBα degradation and NF-κB activation (V. Palombella and Z. Chen, unpubl.).

In this paper we show that deletion of the amino-terminal 36 amino acids of IκBα, or serine to alanine substitutions at either position 32 or 36, block in vitro ubiquitination. Although direct biochemical proof for phosphorylation of IκBα at serine residues 32 and 36 is still lacking, many independent lines of evidence indicate that these two residues are most likely the sites of phosphorylation. First, peptide mapping localizes inducible phosphorylation to the amino terminus of IκBα (Brown et al. 1995). Second, mutants of IκBα containing phosphoserine mimetics (but not alanine) at serine 32 or 36 are competent for degradation in vivo (Brockman et al. 1995). Third, the electrophoretic mobility of mutants containing mimetics at these serine sites coincides with that of the hyperphosphorylated form of endogenous IκBα in activated cells (Fig. 4; data not shown). Fourth, disruption of all other potential phosphorylation sites in the amino terminus of IκBα has no effect on the function of IκBα (Brockman et al. 1995; Brown et al. 1995). Fifth, removal of the carboxy-terminal PEST domain of IκBα fails to prevent inducible hyperphosphorylation in vivo (Brown et al. 1995). Taken together, we propose that phosphorylation of serine residues 32 and 36 targets IκBα to the ubiquitin–proteasome pathway.

The amino terminus of IκBα, which is not required for its association with NF-κB, is highly susceptible to protease cleavage, and this susceptibility is unaffected by binding to the p65 subunit of NF-κB (Jaffray et al. 1995). Thus, the amino terminus of IκBα appears to be exposed in the NF-κB complex and can therefore be recognized by an IκB kinase and presumably ubiquitination enzymes. In contrast, the central region of IκBα, which contains a tandem array of ankyrin repeats, is protease resistant and connected to the acidic carboxy-terminal domain containing a PEST sequence (Jaffray et al. 1995). Recent mutational studies have suggested that this PEST sequence may be required for signal-induced degradation of IκBα

(Miyamoto et al. 1994; Brockman et al. 1995; Brown et al. 1995). The PEST sequences on the cyclin CLN3 protein contain phosphorylation sites, and these sites have been shown to be required for CLN3 degradation during the cell cycle (Yaglom et al. 1995). It should be noted that the ΔC mutant tested in this study lacks the PEST sequence as well as additional carboxy-terminal sequences that are required for binding to NF-κB (Brockman et al. 1995). Although the in vitro-translated ΔC protein is ubiquitinated in vitro, the significance of this observation with respect to the regulated degradation of NF-κB-bound IκBα remains unclear. Notwithstanding this uncertainty, these findings suggest that the PEST sequences of IκBα are not required for ubiquitination of free IκBα.

Investigation of the amino acid sequence requirements for the ubiquitin–proteasome-dependent degradation of other proteins has not revealed a common recognition element (for review, see Ciechanover 1994). For example, a "destruction box" sequence has been shown to be required for the ubiquitin-mediated degradation of mitotic cyclins (Glotzer et al. 1991; Luca et al. 1991). In contrast, a region containing PEST sequences and multiple phosphorylation sites is required for the degradation of CLN3 (Yaglom et al. 1995). In another example, degradation of the transcription factor MATα2 requires two different regions of the protein, and these sequences have not been found in other proteins (Hochstrasser et al. 1991). These and other examples strongly suggest that different protein substrates are recognized for degradation by the ubiquitin–proteasome pathway by distinct mechanisms (Ciechanover 1994). The recognition of specific substrates may involve the use of specific ubiquitin protein ligases (Hershko et al. 1994).

Remarkably, neither phosphorylation nor ubiquitination results in the dissociation of IκBα and NF-κB, and we have shown that the 26S proteasome recognizes and degrades ubiquitinated IκBα in the ternary NF-κB complex. The three-dimensional structure of an NF-κB p50 homodimer bound to DNA has been determined recently (Ghosh et al. 1995; Muller et al. 1995). In addition, specific amino acids in the highly conserved Rel homology domain have been shown to be required for interactions between the *Drosophila* Dorsal and Cactus proteins, homologs of NF-κB and IκB, respectively (Lehming et al. 1995). The location of these amino acids in the three-dimensional structure of the Rel homology domain suggests that IκB may fit within a deep groove formed between the dimerization domain of the two subunits (Ghosh et al. 1995; Lehming et al. 1995; Muller et al. 1995). Thus, IκBα must be phosphorylated and ubiquitinated on the surface away from the dimerization domain, as the modified IκBα remains bound to NF-κB. How, then, is the IκBα degraded as part of the NF-κB complex? An interesting possibility is suggested by the recent observations that molecular chaperones are required for the degradation of certain ubiquitinated substrates by the proteasome (D.H. Lee, M. Sherman, and A.L. Goldberg, pers. comm.). Perhaps, the 26S proteasome binds to the ubiquitin chains on IκBα and, in conjunction with chaperones, strips IκBα away from NF-κB and then unfolds and degrades free IκBα.

We have shown that in vitro-translated IκBα is phosphorylated and ubiquitinated in HeLa cell extracts in the presence of the phosphatase inhibitor okadaic acid. Previous studies have shown that IκBα can be inactivated in vitro by sphingomyelinase or ceramide (Machleidt et al. 1994), LPS (Ishikawa et al. 1995), or ζ protein kinase C (ζPKC) (Diaz-Meco et al. 1994). In contrast, the behavior of the in vitro system described here suggests a low level of constitutive IκBα phosphorylation that would ordinarily not be detected because of the presence of endogenous phosphatases. However, in the presence of okadaic acid, the constitutively phosphorylated IκBα accumulates.

Phosphorylation-dependent ubiquitination of IκBα could occur via two mechanisms, which are not mutually exclusive. First, the phosphorylation of IκBα may enhance its affinity for constitutive ubiquitination enzymes (E2s and E3s). Alternatively, one or more of the enzymes involved in ubiquitination may be activated by the same signal transduction cascade. An example of such regulation is the activation of a cyclin ubiquitin protein ligase (E3) by cdc2 (Hershko et al. 1994).

There are now several examples in which the ubiquitin–proteasome pathway plays an essential role in the regulation of transcription factor levels. These examples include the degradation of yeast MATα2 (Hochstrasser et al. 1991) and GCN4 (Kornitzer et al. 1994) proteins, and the mammalian c-Jun (Treier et al. 1994) and p53 proteins (Scheffner et al. 1993). The example of NFκB1/p105 is exceptional in that the proteasome selectively degrades the carboxyl terminus of an inactive precursor protein, leaving the amino terminus intact (Palombella et al. 1994). The complete degradation of IκBα leads to a rapid and transient activation of NF-κB. The transient nature of the activation is a consequence of the positive autoregulation of the IκBα gene by the activated NF-κB and the subsequent restoration of the cytoplasmic IκBα pool (Sun et al. 1993). In contrast, when the degradation of another IκB protein, IκBβ, is induced by LPS and IL-1, the activation of NF-κB persists (Thompson et al. 1995). The degradation of IκBβ, like that of IκBα, is inhibited by TPCK, which seems to block the activities of one or more IκB kinases. IκBβ contains an amino-terminal sequence strikingly similar to the Ser-32/Ser-36-like region of IκBα (Thompson et al. 1995). Thus, it seems likely that the degradation of IκBβ also involves the ubiquitin–proteasome pathway.

Because of the central role played by NF-κB and other Rel family members in the immune and inflammatory responses, their activation would be an attractive target for the development of pharmacological inhibitors. For example, the genes encoding the cell adhesion molecules expressed on the surface of the vascular endothelium require NF-κB for their induced expression by TNFα and other inflammatory cytokines (for review, see Collins et al. 1995). Recent studies have shown that the proteasome inhibitor MG132 blocks the induction of the leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1 (Read et al. 1995). The functional consequence of this inhibition was the prevention of lymphocyte attachment to TNFα-treated endothelial monolayers. The finding that ubiquitination is required for the proteasome-dependent degradation of IκBα provides additional, and possibly more specific, targets for inhibition of the inflammatory response.

Materials and methods

Materials

The proteasome inhibitor MG132 (Z-Leu-Leu-Leu-H) has been described before (Palombella et al. 1994; Rock et al. 1994). Calyculin A and okadaic acid were purchased from GIBCO BRL. Antibodies against IκBα (c-21, sc-371) and RelA/p65 (sc-109), as well as the agarose conjugates of the RelA antibody (sc-109AC) were purchased from Santa Cruz Biotechnology. Affinity-purified antibody specific for conjugated ubiquitin was provided by Dr. Cecile Pickart (State University of New York, Buffalo). Ubiquitin was purchased from Sigma, and MeUb was prepared according to Hershko and Heller (1985). Fluoresceamine analysis showed that >95% of the lysine residues on MeUb was blocked. Ubal was prepared according to Mayer and Wilkinson (1989). 20S and 26S proteasomes were purified according to published methods (Hough et al. 1987; Ganoth et al. 1988).

Plasmids, in vitro translation, and cell culture

The IκBα mutants are described by Brockman et al. (1995). These mutants were subcloned into pBluescript (SK(+), Stratagene) or pSP72 (Promega) for in vitro translation. Wild-type and mutant IκBα proteins were produced and labeled with [^{35}S]methionine by in vitro translation in TNT wheat germ extracts (Promega) using RNA transcribed from *Not*I linearized plasmids. The translation products were used directly in ubiquitination assays (see below). Jurkat cells (ATCC) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. For metabolic labeling with [^{35}S]methionine/cysteine, 200 μCi/ml of EXPRE^{35}S^{35}S (Dupont NEN) was used in the labeling media lacking methionine and cysteine.

Preparation of cell extracts

Preparation of HeLa cytoplasmic extracts (S100) was described earlier (Fan and Maniatis 1991). These extracts were further concentrated by ammonium sulfate (80%) precipitation, followed by extensive dialysis in 20 mM Tris (pH 7.6), 0.5 mM DTT. The extracts were stored in aliquots at −80°C.

Jurkat cell cytoplasmic extracts were prepared by lysing the cells in a hypotonic buffer (buffer A) containing 10 mM HEPES (pH 7.4), 1 mM EDTA, 10 mM KCl, 1 mM DTT, phosphatase inhibitors (50 mM NaF, 50 mM glycerol-2-phosphate, 1 mM sodium orthovanadate, 0.1 μM okadaic acid), and protease inhibitors (0.1 mg/ml of PMSF, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin). Following incubation on ice for 15 min, 0.2% NP-40 was added to the lysate, and the mixture was placed on ice for another 5 min. After centrifugation at 16,000*g* for 5 min at 4°C, the supernatant (cytoplasmic extract) was stored at −80°C.

Immunoprecipitation and Western blot analysis

Immunoprecipitation was carried out in RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) plus 0.1% SDS. Antibodies were incubated with cytoplasmic extracts at 4°C for 1 hr. Protein A–trisacryl (Pierce) equilibrated in the same buffer was then added to the mixture, and the incubation was continued for another hour. When anti-RelA–agarose was used for immunoprecipitation, the step involving addition of protein A–trisacryl was omitted. After a brief centrifugation, the resin was washed four times with RIPA/0.1% SDS and then boiled in SDS sample buffer.

Coimmunoprecipitations were carried out similar to the immunoprecipitation described above, except that buffer A/0.2% NP-40 (see above) instead of RIPA/0.1% SDS was used for antibody incubations. The resin was then washed with buffer B (10 mM HEPES at pH 7.4, 1 mM EDTA, 10 mM KCl, 50 mM NaF, 50 mM glycerol-2-phosphate, 1 mM sodium orthovanadate, 0.1 mg/ml of PMSF, 0.2% NP-40, and 90 mM NaCl). The washed resin was either boiled in SDS sample buffer, eluted with 0.1 M Capso (pH 11.2), or used directly for assays (see below). Western blot analysis was performed according to Fan and Maniatis (1991).

Ubiquitination assay

In vitro-translated ^{35}S-labeled IκBα was incubated with HeLa extract (4.5 mg/ml) in the presence of an ATP regenerating system (50 mM Tris at pH 7.6, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml of creatine kinase, 0.6 U/ml inorganic pyrophosphatase), together with ubiquitin (1 mg/ml), okadaic acid (3 μM), and Ubal (3 μM). The reactions were incubated at 37°C for 1 hr unless otherwise indicated. After terminating the reaction with SDS sample buffer, the reaction mixture was subjected to SDS-PAGE (9%) and fluorography.

Isolation of ubiquitin-IκBα conjugates

Ubiquitinated IκBα was synthesized in a 300-μl reaction mixture containing 60 μl of in vitro-translated ^{35}S-labeled IκBα, 1.5 mg of HeLa extract, and other components of the ubiquitination reaction. The control reaction contained 40 mM EDTA instead of Mg-ATP in the mixture. After 2 hr of incubation at 37°C, ubiquitinated IκBα and unconjugated IκBα were coimmunoprecipitated with RelA using 30 μl of anti-RelA agarose conjugates (0.25 μg/μl of antibody). Following incubation at 4°C for 1 hr, the resin was washed three times with buffer B and once with buffer D (50 mM Tris at pH 7.6, 0.5 mM DTT). The resin was then resuspended in buffer D and used directly in the conjugate degradation assay.

Conjugate degradation assay

Ubiquitinated IκBα suspension (~2000 cpm) was incubated with 17 nM of 26S proteasome in an ATP-regenerating system (see above). At the desired time points, the reaction was quenched by addition of 125 μl of 4% BSA and 575 μl of 12% TCA. After removal of the TCA precipitates by centrifugation, 600 μl of the supernatants was counted in a scintillation counter. The results are expressed as percentage of the conjugates that are degraded to TCA-soluble counts.

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